EXHIBIT 102

Neurobiology of Aging 35 (2014) 1992–2003

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Altered arginine metabolism in Alzheimer's disease brains

Ping Liu [a,b,*], Michael S. Fleete [a,b], Yu Jing [a,b], Nicola D. Collie [a,b], Maurice A. Curtis [c], Henry J. Waldvogel [c], Richard L.M. Faull [c], Wickliffe C. Abraham [b,d], Hu Zhang [b,e]

[a] Department of Anatomy, University of Otago, Dunedin, New Zealand
[b] Brain Health Research Centre, University of Otago, Dunedin, New Zealand
[c] Centre for Brain Research and Department of Anatomy with Radiology, University of Auckland, New Zealand
[d] Department of Psychology, University of Otago, Dunedin, New Zealand
[e] School of Pharmacy, University of Otago, Dunedin, New Zealand

ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form 27 February 2014
Accepted 14 March 2014
Available online 20 March 2014

Keywords:
Alzheimer's disease
Aging
Nitric oxide synthase
Arginase
Polyamines
Urea cycle

ABSTRACT

L-arginine is a semi-essential amino acid with a number of bioactive metabolites. Accumulating evidence suggests the implication of altered arginine metabolism in the pathogenesis of Alzheimer's disease (AD). The present study systematically compared the metabolic profile of L-arginine in the superior frontal gyrus, hippocampus, and cerebellum from AD (mean age 80 years) and normal (mean age 80 or 60 years) cases. The activity and protein expression of nitric oxide synthase and arginase were altered with AD and age in a region-specific manner. There were also AD- and age-related changes in the tissue concentrations of L-arginine and its downstream metabolites (L-citrulline, L-ornithine, agmatine, putrescine, spermidine, spermine, glutamate, γ-aminobutyric acid, and glutamine) in a metabolite- or region-specific manner. These findings demonstrate that arginine metabolism is dramatically altered in diverse regions of AD brains, thus meriting further investigation to understand its role in the pathogenesis and/or progression of the disease.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative condition with memory loss as an early prognostic sign and aging as a major risk factor. Its cardinal histopathological features consist of aggregates of hyperphosphorylated tau and amyloid beta (Aβ), which form intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques (SPs), respectively, in the affected brain regions. Although Aβ plays a central and causative role in the disease development (Hardy and Higgins, 1992; Selkoe, 2011), a growing body of evidence suggests the involvement of arginine metabolism in AD pathogenesis (Law et al., 2001; Malinski, 2007; Yi et al., 2009).

L-arginine is a semi-essential amino acid that can be metabolized to form a number of bioactive molecules (Fig. 1; Wu and Morris, 1998). Nitric oxide (NO) is a gaseous signaling molecule produced by NO synthase (NOS). NO derived from neuronal NOS (nNOS) plays an important role in synaptic plasticity and learning and memory (Feil and Kleppisch, 2008; Susswein et al., 2004; Zhou and Zhu, 2009), whereas endothelial NOS (eNOS)-derived NO is a key factor for the stabilization and regulation of the vascular microenvironment (de la Torre, 2012; Forstermann and Sessa, 2012). In AD brains, NFTs and SPs are associated with reduced capillary expression of eNOS (Jeynes and Provias, 2009; Provias and Jeynes, 2008). There is evidence suggesting that eNOS-derived NO can directly modulate the production of Aβ and protect against increases in Aβ (Austin et al., 2010). Because of its nature as a free radical, however, an excessive amount of NO, particularly that derived from inducible NOS (iNOS), leads to neurotoxicity and neurodegeneration (Law et al., 2001; Malinski, 2007). It has been reported that NO produced in response to Aβ triggers mitochondrial fission, synaptic loss, and neuronal damage (Cho et al., 2009).

L-ornithine is the arginase-mediated metabolite of L-arginine, with urea as the by-product (Fig. 1). It can be metabolized by ornithine decarboxylase (ODC) to produce the polyamines putrescine, spermidine, and spermine (Fig. 1), which are essential for cells to grow and to function in an optimal manner (Alm and Oredsson, 2009; Igarashi and Kashiwagi, 2010; Wallace, 2009; Wallace et al., 2003). In AD brains, there is altered arginase I and arginase II messenger RNA (mRNA) expression, ODC protein expression, and polyamine tissue concentrations (Colton et al., 2006; Hansmannel et al., 2010; Morrison and Kish, 1995; Morrison et al., 1998). Moreover, the arginase II allele rs742869 is associated with an increased risk of earlier onset AD (Hansmannel et al., 2010). L-ornithine can also be channeled to produce glutamate that can be

* Corresponding author at: Department of Anatomy, University of Otago, Dunedin 9016, New Zealand. Tel.: +(64) 03 4797536; fax: +(64) 03 4797254.
E-mail address: ping.liu@otago.ac.nz (P. Liu).

0197-4580/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2014.03.013

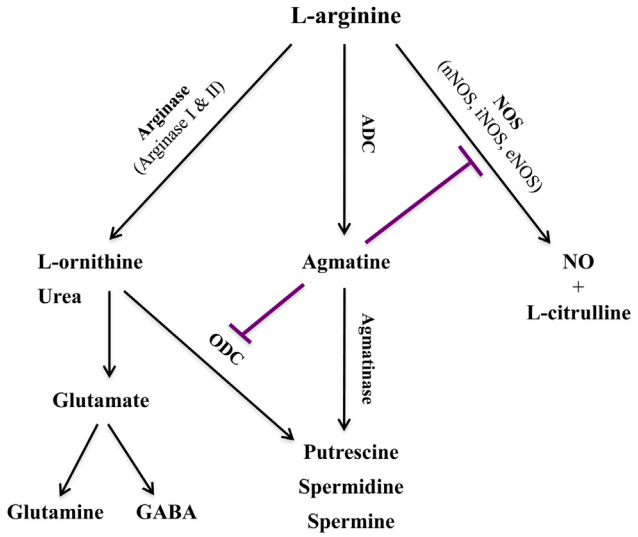

Fig. 1. Arginine metabolic pathways. L-arginine can be metabolized by nitric oxide synthase (NOS), arginase, and arginine decarboxylase (ADC) to form a number of bioactive molecules (see text for detailed description). Flat arrowheads indicate the modulation by agmatine on NOS and ornithine decarboxylase (ODC). Abbreviations: eNOS, endothelial nitric oxide synthase; GABA, γ-aminobutyric acid; iNOS, inducible NOS; nNOS, neuronal NOS; NO, nitric oxide.

further metabolized to generate γ-aminobutyric acid (GABA) and glutamine by glutamic acid decarboxylase and glutamine synthase (GS), respectively (Fig. 1; Tapiero et al., 2002; Wiesinger, 2001; Wu and Morris, 1998). Previous research has reported decreased glutamate and GABA levels in AD brains and increased GS level in lumbar cerebrospinal fluid of AD patients (Ellison et al., 1986; Tumani et al., 1999).

Agmatine is the product of arginine decarboxylase (ADC) (Wu and Morris, 1998), and is present in mammalian brain (Li et al., 1994). Agmatine inhibits nNOS and iNOS, but stimulates eNOS, hence it has an important role in regulating NO production (Halaris and Plietz, 2007; Joshi et al., 2007; Santhanam et al., 2007; Satriano, 2003). As agmatine can itself be metabolized by agmatinase to form putrescine (Fig. 1) and induce antizyme, a small regulatory protein that inhibits ODC and down-regulates polyamine uptake (Satriano, 2003), it has an important role in controlling cellular polyamine content. Moreover, agmatine is considered a novel putative neurotransmitter (Reis and Regunathan, 2000), and recent evidence suggests its involvement in learning and memory processes (Leitch et al., 2011; Liu et al., 2008b, 2009a; Rushaidhi et al., 2013; Seo et al., 2011). To the best of our knowledge, there is no previous research on how agmatine changes in AD brains.

Metabolomics refers to the analysis of the component small molecules produced by a biological system and is playing an increasingly prominent role in efforts at biomarker identification for AD (Trushina and Mielke, 2013; Trushina et al., 2013). As described previously, L-arginine is versatile metabolically but its involvement in AD is largely based on scattered information from a single pathway. Hence, it is essential to understand how the arginine metabolic profile changes in AD brains. In the present study, we systematically compared NOS and arginase activity and protein expression, as well as the tissue concentrations of L-arginine and its 9 downstream metabolites in postmortem superior frontal gyrus, hippocampus, and cerebellum from AD cases (mean age 80 years) and normal control cases (mean age 60 or 80 years). This experimental design allowed us to assess the effects of AD, as well as advanced aging, on arginine metabolism in the brain. Since the hippocampus and superior frontal gyrus are more vulnerable in AD, whereas the cerebellum is less affected (Braak and Braak, 1991; Serrano-Pozo et al., 2011), we investigated whether the L-arginine metabolic profile changes are region-specific.

2. Methods

2.1. Human samples

Human brain tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank. All tissue collection protocols were approved by the University of Auckland Human Participants Ethics Committee, and informed consent was obtained from all families. The unfixed snap-frozen superior frontal gyrus (SFG; central portion), hippocampus (HPC; anterior portion), and cerebellum (CE; anterior lobe) were obtained from 11 neurologically normal cases with an average age of 60 years (NC-60; Table 1), 12 neurologically normal cases with an average age of 80 years (NC-80; Table 2), and 12 AD cases with an average age of 80 years (AD-80; Table 3) (for a detailed description regarding the human brain tissue blocking see Waldvogel et al., 2008). The neurologically normal cases were defined by an absence of any history of neurologic and psychiatric disease and the absence of any neuropathological findings from a detailed analysis of the brain by an independent neuropathologist. AD cases were assessed using the Consortium to Establish a Registry for Alzheimer Disease probability indexing. Of the 12 cases, 4 were definite AD, 6 were probable AD, and 2 were possible AD (Table 3). There were 58.3% men in the NC-80 and AD-80 groups and 90.9% of men in the NC-60 group, with no significant differences between groups in the postmortem delay or storage time (Tables 1–3). The experimenters were blind to the grouping information.

Table 1
Neurologically normal cases with an average age of 60 years (NC-60)

Case number	Gender	Age (y)	Postmortem delay (h)	Storage time (mo)	Cause of death
H108	Male	58	16	235	Coronary atherosclerosis
H115	Male	61	12	138	Hypertensive heart disease
H118	Male	57	10	134	Coronary artery disease
H119	Male	58	15	132	Ischaemic heart disease
H125	Male	58	13	136	Coronary artery disease
H127	Female	59	21	131	Pulmonary embolus
H155	Male	61	7	81	Ischaemic heart disease
H170	Male	60	17	62	Ischaemic heart disease
H174	Male	59	24.5	60	Aortic aneurysm
H200	Male	56	23	36	Asphyxia
H204	Male	66	9	32	Ischaemic heart disease
Mean ± SEM		59.4 ± 0.8	15.3 ± 1.7	107 ± 18	

Key: SEM, standard error of the mean.

Table 2
Neurologically normal cases with an average age of 80 years (NC-80)

Case number	Gender	Age (y)	Postmortem delay (h)	Storage time (mo)	Cause of death
H85	Male	94	10	211	Myocardial infarction
H109	Male	81	7	162	Coronary atherosclerosis
H110	Female	83	14	162	Ruptured aortic aneurysm
H112	Male	79	8	154	Bleeding stomach ulcer
H122	Female	72	9	129	Emphysema
H123	Male	78	7.5	129	Ruptured abdominal aortic aneurysm
H137	Female	77	12	101	Coronary atherosclerosis
H150	Male	78	12	87	Ruptured myocardial infarction
H169	Male	81	24	63	Asphyxia
H181	Female	78	22	49	Aortic aneurysm
H190	Female	72	19	42	Ruptured myocardial infarction
H202	Male	83	14	35	Ruptured abdominal aortic aneurysm
Mean ± SEM		79.7± 1.7	13.3 ± 1.7	110.3 ± 16.3	

Key: SEM, standard error of the mean.

2.2. Tissue preparation

The SFG, HPC, or CE tissue (in sections) from each case was mixed and then divided into 2 parts. Protease-inhibitory buffer containing 50 mM Tris-HCl (pH 7.4), 10 μM phenylmethylsulfonyl fluoride, 15 μM pepstatin A, and 2 μM leupeptin (1:10 wt/vol) was added to the first part of tissue on ice, and the samples were then homogenized using ultrasonification (Branson Sonifier 150D, Branson Ultrasonics Corporation, Danbury, CT, USA) and centrifuged at 12,000 *g* for 10 minutes at 4 °C. Protein concentrations in the supernatant were measured based on the Bradford method (Bradford, 1976) using a Bio-Rad protein assay dye reagent concentrate and Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories Inc, Hercules, CA, USA) (Knox et al., 2011; Liu et al., 2005, 2009b, 2011). Each supernatant was then aliquoted and used for the assays and Western blot. The second part of tissue from each case was weighed, homogenized in ice-cold 10% perchloric acid (~50 mg wet weight per milliliter) and centrifuged at 10,000 rpm for 10 minutes to precipitate protein. The supernatants (the perchloric acid extracts) were frozen immediately and stored at −80 °C for later high performance liquid chromatography and liquid chromatography/mass spectrometric assays.

2.3. NOS and arginase assays

We used a radioenzymatic assay technique (Bredt and Snyder, 1990), to analyze NOS activity by measuring the ability of tissue homogenates to convert [^{3}H] L-arginine to [^{3}H] L-citrulline in the presence of co-factors, and a spectrophotometric assay method to determine arginase activity by measuring the amount of newly formed urea from L-arginine, as described in our previous publications (Gupta et al., 2012; Liu et al., 2003a, 2003b, 2005, 2009b). The contribution of iNOS (calcium-independent) to total NOS activity was assessed in the absence of calcium. All assays were performed in triplicate. For each brain region, the samples from all 3 groups were processed at the same time and the order was counterbalanced. NOS and arginase activities were expressed as pmol [^{3}H] L-citrulline/30 min/mg protein and μg urea/mg protein, respectively.

We further measured the levels of nitrate and nitrite (NOx, the end products of NO) in the superior frontal gyrus and cerebellum (hippocampal tissue being unavailable) using the Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Ilc, Oxford, MI, USA) based on the manufacturer's instructions. Total nitrate and/or nitrite concentration in each sample (supernatant) was determined via a 2-step process, the conversion of nitrate to nitrite using nitrate reductase followed by quantization of nitrite using Griess Reagent. For each brain region, the samples from all 3 groups were processed at the same time in duplicates and the order was counterbalanced. The NOx level was expressed as nmol nitrite/mg protein.

2.4. Western blotting

The protein expression of nNOS, eNOS, iNOS, arginase I, arginase II, and β-actin in each sample was determined using the Western blot method. The protein concentrations in all the brain samples were equalized to 2 (for nNOS, eNOS, arginase I, and arginase II) or 8 (for iNOS) mg/mL. The supernatants were then mixed with gel loading buffer (50 mM Tris-HCl, 10% sodium dodecyl sulfate, 10%

Table 3
Alzheimer's disease cases with an average age of 80 years (AD-80)

Case number	Gender	Age (y)	Postmortem delay (h)	Storage time (mo)	Cause of death	Duration of symptoms (y)	CERAD
AZ 32	Female	75	3	160	Bronchiectasis pneumonia	Years	Probable Alzheimer's
AZ 37	Male	83	4	126	Bronchopneumonia	12	Probable Alzheimer's
AZ 38	Male	80	5.5	125	Respiratory arrest	15–20	Definite Alzheimer's
AZ 43	Male	80	21	114	Bronchopneumonia	17 (main symptoms 7)	Probable Alzheimer's
AZ 45	Male	82	4.5	120	Pneumonia	10	Probable Alzheimer's
AZ 46	Female	82	22	119	Coronary artery disease	18 mo	Probable Alzheimer's
AZ 54	Male	84	3	106	Bronchopneumonia	Years	Possible Alzheimer's
AZ 59	Male	83	15	91	Cardiopulmonary collapse	5	Possible Alzheimer's
AZ 65	Female	77	16	61	Bronchopneumonia	14	Definite Alzheimer's
AZ 74	Female	85	16	38	Aspiration pneumonia	5	Definite Alzheimer's
AZ 77	Female	81	16	30	Bronchopneumonia	Years	Probable Alzheimer's
AZ 80	Male	77	4.5	22	Myocardial infarction	10	Definite Alzheimer's
Mean ± SEM		80.8 ± 0.9	10.9 ± 2.1	92.7 ± 12.8			

Key: CERAD, Consortium to Establish a Registry for Alzheimer's Disease; SEM, standard error of the mean.

glycerol, 10% 2-mercaptoethanol, 2 mg/mL bromophenol blue) in a ratio of 1:1 and then boiled for 5 minutes. Ten μL of each sample was loaded in each well on a precast 4%–12% Bis-Tris Criterion gel (Bio-Rad), and a pre-stained protein marker (41.5–203 kDa; Bio-Rad) was run on the same gel. The proteins were transferred to polyvinylidene-difluoride membranes using a transblotting apparatus (Bio-Rad). The transfer was performed overnight in transfer buffer (25% methanol, 1.5% glycine, and 0.3% Tris-base). Nonspecific IgG binding was blocked by incubation with 5% dried milk protein and 0.1% bovine serum albumin for at least 7 hours. The membranes were then incubated with an affinity-purified monoclonal mouse antibody raised against nNOS (Santa Cruz Biotechnology, Dallas, Texas, sc-5302, 1:5000) or arginase I (Santa Cruz Biotechnology, sc-166920, 1:1000), or polyclonal rabbit antibody raised against eNOS (Santa Cruz Biotechnology, sc-653, 1:10,000), iNOS (Santa Cruz Biotechnology, sc-651, 1:1000) or arginase II (Santa Cruz Biotechnology, sc-20151, 1:1000) overnight at 4 °C. The secondary antibody was an anti-mouse (Santa Cruz Biotechnology, sc-2005, 1:5000) or an anti-rabbit (Santa Cruz Biotechnology, sc-2004, 1:10,000 for eNOS, 1:1000 for iNOS and arginase II) IgG linked to horseradish peroxidase. To ensure that the same amount of protein was loaded in each lane, an IgG monoclonal antibody against β-actin (Santa Cruz Biotechnology, sc-47778, 1:200,000; secondary: goat anti-mouse IgG, Santa Cruz Biotechnology, sc-2005, 1:20,000) was used as a loading control. Immuno-detection was performed using the enhanced chemiluminescence system (Amersham Biosciences, Auckland, NZ). Hyperfilms (Amersham Biosciences, NZ) were analyzed by densitometry to determine the quantity of protein expressed in each group using the Bio-Rad Quantity One software (Knox et al., 2011; Liu et al., 2011). Results were expressed as volume of the band (optical density × area of the band) and normalized by the corresponding β-actin loading controls.

2.5. Immunohistochemistry

Immunohistochemistry on free-floating formalin-fixed superior frontal cortex and cerebellum sections from the AD-80 (n = 3; AZ38, AZ77, and AZ80) and NC-80 (n = 3; H137, H169, and H202) groups was performed as detailed in Waldvogel et al. (2006). Briefly, 50-μm-thick tissue sections were permeabilized in 0.1 M sodium citrate buffer (pH 4.5) overnight at 4 °C followed by antigen retrieval using microwave for 30 seconds. Endogenous peroxidase activity was blocked with 1% H_2O_2 in 50% methanol for 20 minutes at room temperature. Sections were incubated with polyclonal rabbit iNOS antibody (sc-651, 1:100) for 3 days at 4 °C followed by the incubation with the biotinylated secondary antibody (Dako kit) overnight at room temperature. To increase the sensitivity of the detection, sections were incubated with streptavidin peroxidase complex (Dako kit) for 4 hours at room temperature. The sections were then reacted in 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma, Sydney, Australia) and 0.01% H_20_2 in 0.1 M phosphate buffer (pH 7.4) for 10–20 minutes to produce a brown reaction product and mounted in gelatin, dehydrated, and cover slipped with DPX mounting medium (Merck). A no-primary antibody condition was used as a negative control.

2.6. Amino acid and polyamine analyses

Determination of amino acids (L-arginine, L-citrulline, L-ornithine, glutamine, glutamate, and GABA) and polyamines spermidine and spermine were carried out by the high performance liquid chromatography methods, and agmatine and putrescine levels were measured by a highly sensitive liquid chromatography/mass spectrometric method, as we have previously described (Gupta et al., 2012; Liu et al., 2008a, 2008b, 2008c, 2009a, 2009b, 2010, 2011). For each brain region, samples from the 3 groups were assayed at the same time in a counterbalanced manner, and the assays were performed in triplicate. High purity external and internal standards were used (Sigma). All other chemicals were of analytical grade. The concentrations of L-arginine, L-citrulline, L-ornithine, glutamine, glutamate, GABA, agmatine, putrescine, spermidine, and spermine in tissue were calculated with reference to the peak area of external standards and values were expressed as μg/g wet tissue.

2.7. Statistical analysis

Data were analyzed using 1-way analysis of variance followed by Bonferroni post hoc tests (Zolman, 1993). Significance was set at $p < 0.05$ for all comparisons. All calculations were performed with the Prism statistics program and only the significant statistics are reported.

3. Results

3.1. AD- and age-related changes in NOS activity and protein expression

The present study used the radioenzymatic assay to determine the total NOS (in the presence of co-factors) and iNOS (in the absence of calcium) activities in the superior frontal gyrus, hippocampus, and cerebellum across the 3 groups. The total NOS activity was significantly different between groups in SFG ($F = 15.97$, $p < 0.0001$), HPC ($F = 22.79$, $p < 0.0001$), and CE ($F = 6.04$, $p < 0.001$), with markedly reduced levels in the AD-80 group relative to the NC-60 (all 3 regions, 60%–94% decrease) and NC-80 (SFG and HPC, 68%–89% decrease) groups (Fig. 2A). There were also reduced total NOS activities in the NC-80 group when compared with the NC-60 one (HPC and CE, 60%–65% decrease; Fig. 2A). However, iNOS activity was not detectable in any brain region examined. When the levels of nitrate and nitrite (the end products of NO) were measured using the colorimetric assay, there was no significant difference between groups in SFG or CE (Fig. 3A).

We then investigated how the protein expression of NOS changed in each brain region across the 3 groups. Western blot revealed nNOS, eNOS, and iNOS protein at 155, 140, and 130 kDa, respectively. We found significant group differences in nNOS protein expression in SFG ($F = 18.04$, $p < 0.0001$), HPC ($F = 22.30$, $p < 0.0001$), and CE ($F = 8.44$, $p < 0.01$), with lower levels in the AD-80 group relative to the NC-60 (all 3 regions, 70%–90% decrease) and NC-80 (HPC only, 78% decrease). There were also reduced nNOS protein levels in the NC-80 group when compared with the NC-60 one (all 3 regions, 50%–75% decrease; Fig. 2B and G). For eNOS, significant group differences were noticed in SFG ($F = 13.02$, $p < 0.001$) and HPC ($F = 7.91$, $p = 0.003$), but not CE. Post hoc tests indicated lower levels of eNOS protein in the AD-80 group relative to the NC-60 (SFG and HPC, 92%–96% decrease) and NC-80 (SFG and HPC, 70%–94% decrease) groups, with no significant differences between the latter 2 (Fig. 2C and G). Furthermore, we found a significant group difference in iNOS protein expression in SFG ($F = 20.06$, $p < 0.0001$), but not CE, with lower levels in the AD-80 group relative to the NC-60 (about 70% decrease) and NC-80 (about 30% decrease) groups, and in the NC-80 group when compared with the NC-60 one (about 50% decrease) (Fig. 3B and C).

3.2. iNOS immunohistochemical labeling

As iNOS activity was not detectable in the 3 regions examined, we carried out an immunohistochemistry experiment to determine its presence and to compare its immunoreactivity in SFG and CE

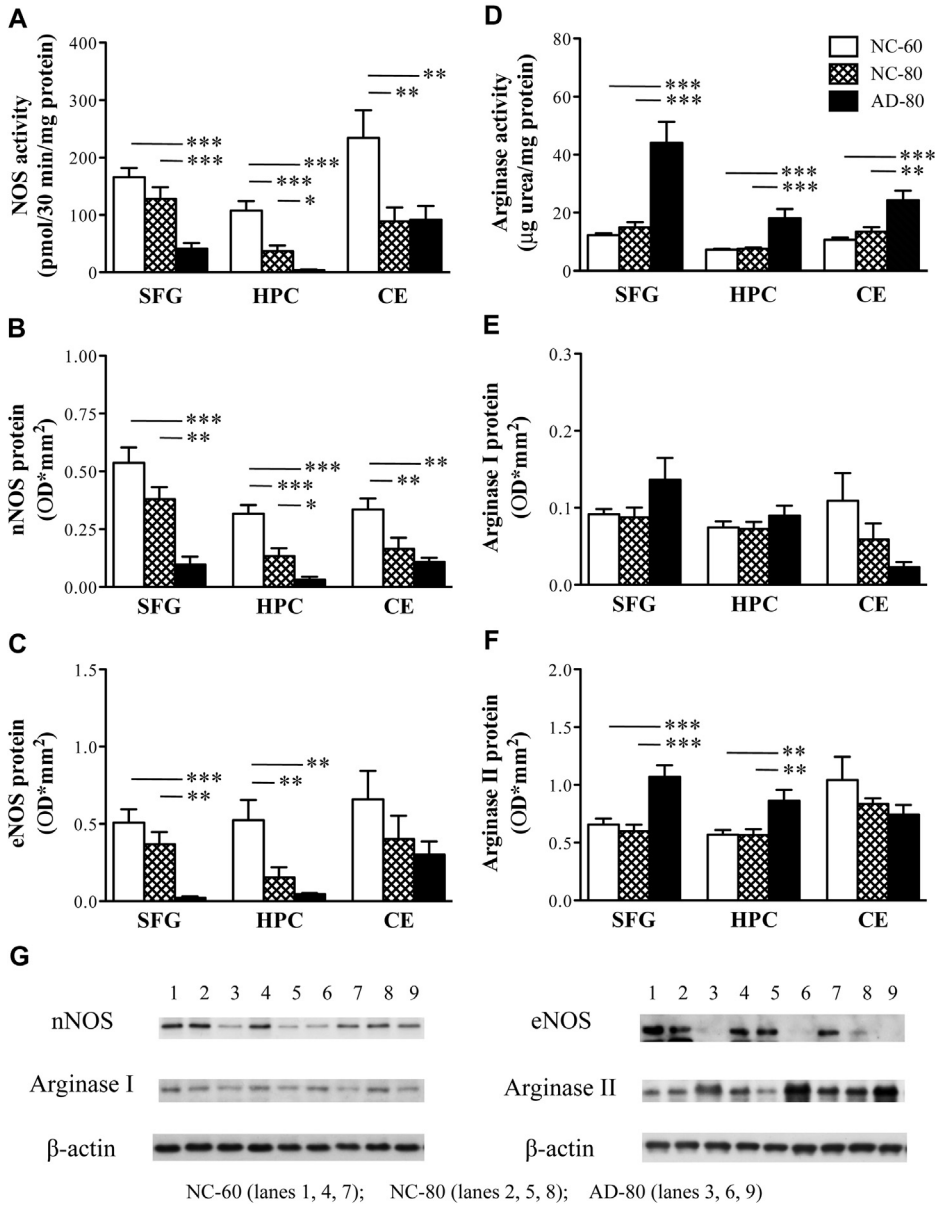

Fig. 2. Mean (±SEM) total NOS (A) and arginase (D) activity, and protein levels of nNOS (B), eNOS (C), arginase I (E) and arginase II (F) in the superior frontal gyrus (SFG), hippocampus (HPC), and cerebellum (CE) from neurologically normal cases with an average age of 60 (NC-60) or 80 (NC-80) years, or Alzheimer's disease cases with an average age of 80 years (AD-80). (G) Example of Western blots showing nNOS, eNOS, arginase I, and arginase II protein expression in SFG across the 3 groups with their corresponding β-actin loading controls. Asterisks indicate significant differences between groups at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. Abbreviation: SEM, standard error of the mean.

between the NC-80 and AD-80 groups. In SFG, iNOS immunoreactivity was observed in neurons with moderate intensity in the NC-80 group, and the immunoreactive astrocytes were seen occasionally (Fig. 4A). In the AD-80 group, by contrast, there were intensely iNOS-immunoreactive neurons and astrocytes in SFG, with some neurons displaying markedly enhanced staining intensity and astrocytes forming clusters (Fig. 4B). In CE, iNOS-immunoreactivity was found in the Purkinje cells and endothelial cells of blood vessels in both groups, with no marked immunoreactive astrocytes (Fig. 4C and D).

3.3. AD-related changes in arginase activity and protein expression

The spectrophotometric assay determined significant group differences in SFG ($F = 16.06$, $p < 0.0001$), HPC ($F = 14.13$, $p < 0.0001$), and CE ($F = 10.49$, $p = 0.0003$) in terms of the total arginase activity, with higher levels in the AD-80 group relative to the NC-60 and NC-80 groups (all 3 regions, 130%–260% and 80%–195% increase, respectively) and no differences between the 2 NC groups (Fig. 2D). To further understand which isoform of arginase contributed to the previously mentioned activity changes, Western blot was performed and a band at 37 kDa was detected for both arginase I and arginase II. The protein level of arginase I was not statistically different between groups in either brain region examined (Fig. 2E and G). For arginase II, however, there were significant group differences in SFG ($F = 12.16$, $p = 0.0001$) and HPC ($F = 6.44$, $p = 0.0047$), but not CE. Post hoc tests revealed higher levels of arginase II protein in the AD-80 group relative to the 2 NC groups (SFG and HPC, 50%–80% increase) with no difference between the latter 2 (Fig. 2F and G). These

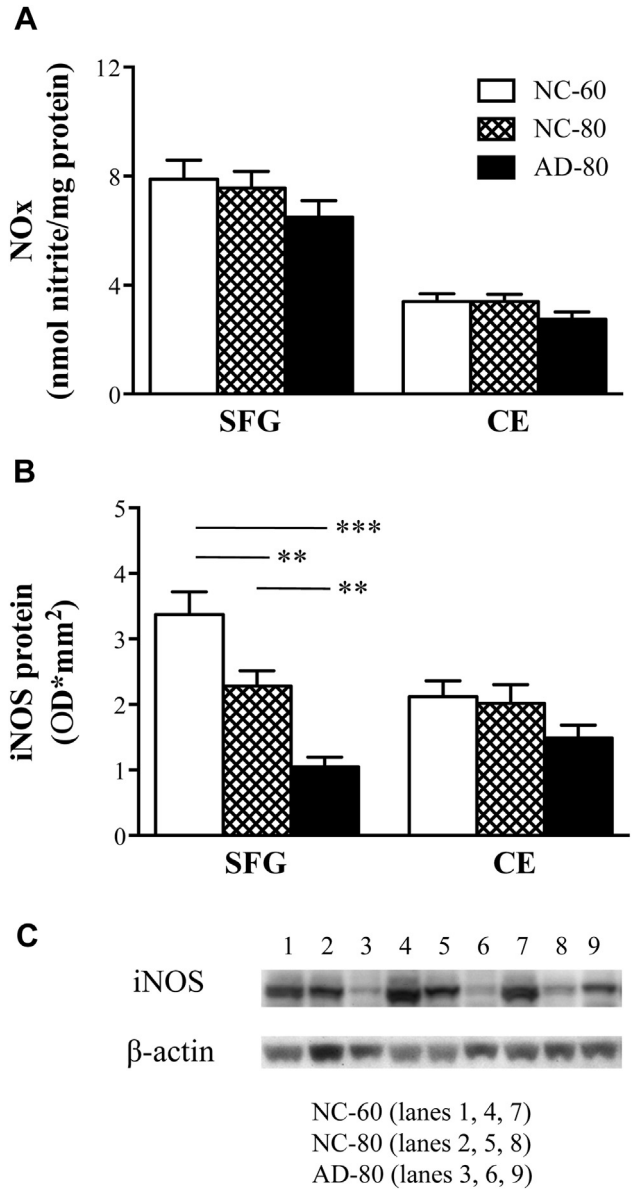

Fig. 3. Mean (±SEM) NOx levels (A) and protein levels of iNOS (B) in the superior frontal gyrus (SFG) and cerebellum (CE) from neurologically normal cases with an average age of 60 (NC-60) or 80 (NC-80) years, or Alzheimer's disease cases with an average age of 80 years (AD-80). (C) Example of Western blots showing iNOS protein expression in SFG across the 3 groups with their corresponding β-actin loading controls. Asterisks indicate significant differences between groups at ** $p < 0.01$ or *** $p < 0.001$. Abbreviations: iNOS, inducible nitric oxide synthase; NOx, nitrate and nitrite; SEM, standard error of the mean.

results suggest that arginase II may be responsible for the increased total arginase activity in the AD-80 group in SFG and HPC. However, there were no alterations in arginase I and arginase II protein expression that could explain the activity change in the AD-80 group in CE.

3.4. AD- and age-related changes in tissue concentrations of L-arginine and its downstream metabolites

Fig. 5 illustrates the tissue concentrations of 6 amino acids in the superior frontal gyrus, hippocampus, and cerebellum across the 3 groups. L-arginine was significantly different between groups in SFG ($F = 6.83$, $p = 0.003$), but not HPC or CE, with a higher level in the AD-80 group relative to the NC-60 (110% increase) and NC-80 (45% increase) groups and no significant difference between the latter 2 (Fig. 5A). For L-ornithine, significant group differences were found in SFG ($F = 23.04$, $p < 0.0001$), HPC ($F = 39.61$, $p < 0.0001$), and CE ($F = 10.97$, $p = 0.0002$) with lower levels in the AD-80 group when compared with the 2 NC groups (all 3 regions, 45%–70% decrease) and no difference between the latter 2 (Fig. 5C). The glutamine levels were significantly different between groups in SFG ($F = 8.01$, $p = 0.0015$) and CE ($F = 7.49$, $p = 0.002$), but not in HPC, with higher levels in the AD-80 group when compared with the NC-60 and NC-80 groups (SFG and CE, 40% increase) and no difference between the latter 2 (Fig. 5D). We also found significant group differences in glutamate in HPC ($F = 4.47$, $p = 0.019$) and CE ($F = 7.58$, $p = 0.002$), but not in SFG, with a lower level in the AD-80 group relative to the NC-60 group in HPC (12% decrease) and a higher level in the AD-80 group when compared with the 2 NC groups in CE (10%–14% increase; Fig. 5E). There was no significant difference between groups in L-citrulline (Fig. 5B) or GABA (Fig. 5F) in any region examined.

Fig. 6 presents the concentrations of agmatine, putrescine, spermidine, and spermine in each brain region across the 3 groups. We found significant differences between groups in agmatine in SFG ($F = 8.52$, $p = 0.001$) and CE ($F = 12.95$, $p < 0.0001$), but not in HPC, with lower levels in the NC-80 and AD-80 groups relative to the NC-60 group (SFG and CE, 25%–40% decrease) and no significant difference between the former 2 (Fig 6A). For putrescine, there were significant group differences in SFG ($F = 13.22$, $p < 0.0001$), HPC ($F = 14.69$, $p < 0.0001$), and CE ($F = 11.23$, $p = 0.0002$), with lower levels in the AD-80 group relative to the NC-60 (SFG and HPC, 50%–70% decrease; CE, 25% decrease) and NC-80 (SFG and HPC, 40%–50% decrease) groups. We also found reduced putrescine levels in the NC-80 group when compared with the NC-60 group in all 3 regions examines (20%–40% decrease; Fig. 6B). The spermidine level was significantly different between groups in SFG only ($F = 6.75$, $p = 0.004$), with a lower level in the AD-80 group relative to the 2 NC groups (30%–40% decrease) and no difference between the latter 2 (Fig. 6C). For spermine, the only significant group difference was noticed in HPC ($F = 5.69$, $p = 0.008$), with a 20% reduction in the NC-80 group when compared with the NC-60 and AD-80 groups and no difference between the latter 2 (Fig. 6D).

4. Discussion

L-arginine is a versatile amino acid that can be metabolized by NOS, arginase, and ADC to form a number of bioactive molecules (Wiesinger, 2001; Wu and Morris, 1998). The present study, for the first time, systematically compared its metabolic profile in the superior frontal gyrus, hippocampus, and cerebellum in the neurologically normal cases with an average age of 60 (NC-60) or 80 (NC-80) years and AD cases with an average age of 80 years (AD-80). Because there were no significant differences between groups in the postmortem delay and tissue storage time, any difference observed should not be attributed to the quality of tissue. As the main objective of the present study was to assess the effects of AD on arginine metabolism, both age and gender were matched between the AD-80 and NC-80 groups.

4.1. AD- and age-related changes in NOS and arginase

We first measured the activity and protein expression of NOS using the radioenzymatic assay and Western blot. There were significantly decreased total NOS activity (in SFG and HPC) and reduced protein expression of nNOS (in SFG and HPC), eNOS (in SFG), and iNOS (in SFG; HPC not assessed) in the AD-80 group relative to the 2 NC groups. There were also age-related decreases in

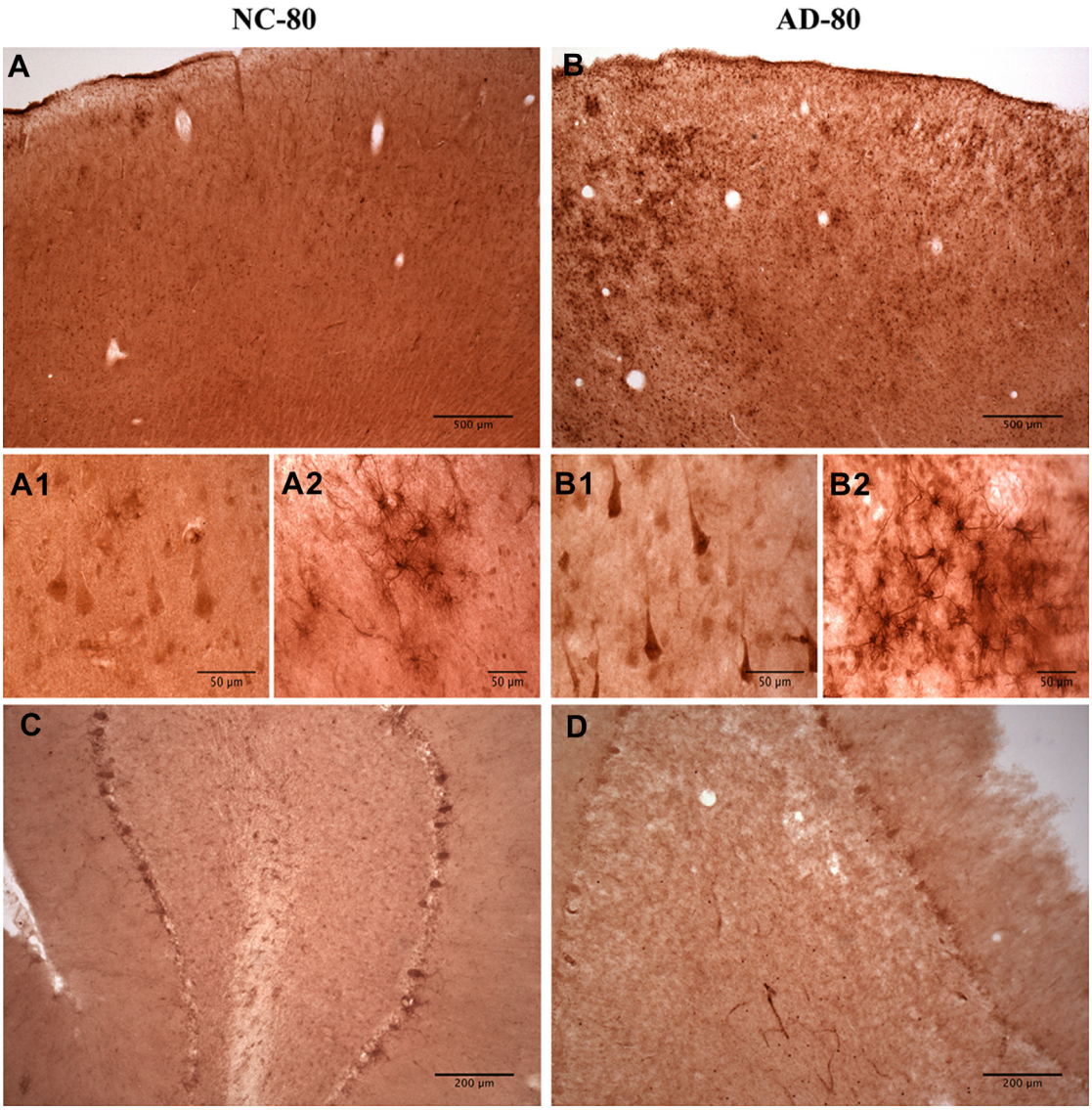

Fig. 4. Immunoreactivity for iNOS in the superior frontal cortex (A and B) and cerebellum (C and D) from neurologically normal cases (NC-80; A and C) and Alzheimer's disease cases (AD-80; B and D) with an average age of 80 years. In the superior frontal cortex, iNOS immunoreactivity was observed in neurons with moderate intensity (A1) and astrocytes (A2) occasionally in the NC-80 group. By contrast, there were intense iNOS-immunoreactive neurons (B1) and clustered astrocytes (B2). In the cerebellum, iNOS-immunoreactivity was found in the Purkinje cells in both groups. Abbreviation: iNOS, inducible nitric oxide synthase.

total NOS activity (in HPC and CE) and nNOS (in HPC and CE), eNOS (in HPC), and iNOS (in SFG) protein expression. Since iNOS activity was not detectable in any region examined under the present experimental conditions, the constitutive forms of NOS (nNOS and eNOS) perhaps make major contributions to the previously mentioned AD- and age-related changes in total NOS activity. A number of earlier studies have reported reduced number of nNOS-positive neurons, decreased nNOS mRNA expression, and negative correlations between the Braak and Braak stage and the number of nNOS-containing neurons, in the AD frontal and entorhinal cortices and hippocampus (Norris et al., 1996; Thorns et al., 1998; Yew et al., 1999) suggesting the vulnerability of nNOS neurons in AD. Earlier research has also shown reduced capillary eNOS expression in the AD superior temporal gyrus and calcarine cortex and the negative correlations with the density of NFTs and SPs (Jeynes and Provias, 2009; Provias and Jeynes, 2008), indicating endothelial hypofunction in AD brains. Collectively, the existing evidence strongly supports the implication of altered nNOS and eNOS function in the pathogenesis of AD.

The radioenzymatic assay employed in the present study measured the ability of tissue homogenates (supernatants) to convert [^{3}H] L-arginine to [^{3}H] L-citrulline in the presence of co-factors. It is currently unclear, however, how the enzymatic activity of each NOS isoform in postmortem tissue is affected by the long-term storage. Nitrate and nitrite (NOx) are the stable end products of NO, and their level has been used as an index of NO production (Sun et al., 2003). Hence, the ultrasensitive colorimetric NOS assay was performed to compare the NOx level in SFG and CE (HPC tissue not available) across the 3 groups. Surprisingly, the NOx level was not statistically different between groups in both SFG and CE, which appear to contradict with the AD- and/or age-related decreases in the total NOS activity in these regions determined by the radioenzymatic assay. One possible explanation for such insignificant changes in NOx could be more nitrate and nitrite produced endogenously (particularly from iNOS) in the NC-80 and AD-80 groups. However, reduced or unchanged iNOS protein level with AD or age does not support the idea. It has been shown that NOx can be sourced from the diet, in addition to endogenous

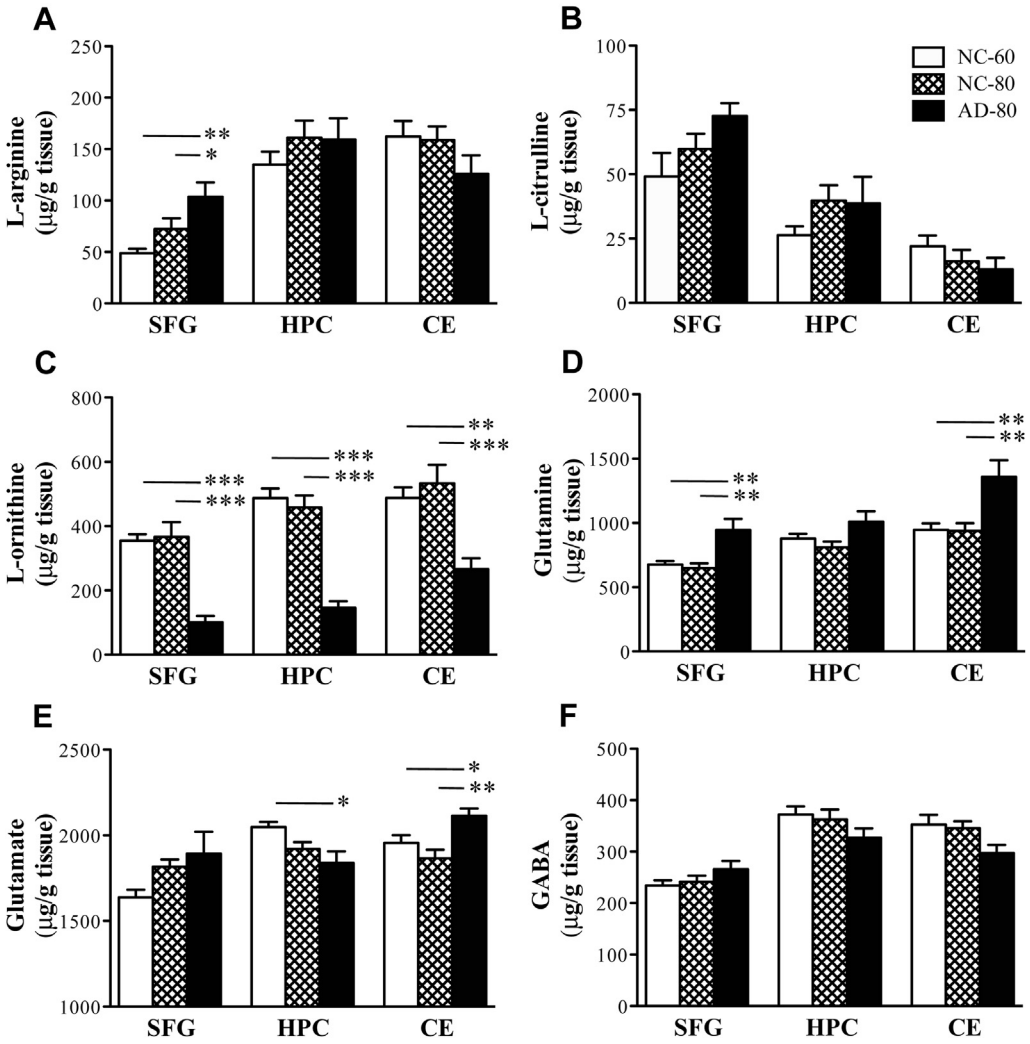

Fig. 5. Mean (±SEM) L-arginine (A), L-citrulline (B), L-ornithine (C), glutamine (D), glutamate (E) and GABA (F) levels in the superior frontal gyrus (SFG), hippocampus (HPC), and cerebellum (CE) from neurologically normal cases with an average age of 60 (NC-60) or 80 (NC-80) years, or Alzheimer's disease cases with an average age of 80 years (AD-80). Asterisks indicate significant differences between groups at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. Abbreviations: GABA, γ-aminobutyric acid; SEM, standard error of the mean.

L-arginine-NO synthase pathway (Lundberg et al., 2008). Milsom et al. (2012), for example, demonstrated that dietary NOx intake restriction resulted in a significant reduction in both the nitrate and nitrite levels in the rat brains, suggesting the contribution of dietary NOx to the brain NOx. Since the blood brain barrier is dysfunctional during AD and aging (Marques et al., 2013), there may be increased dietary and/or peripheral source of NOx in AD and advanced aged brains.

It has been documented that iNOS is present in AD brains, particularly in the NFT-containing neurons and astrocytes surrounding SPs (Luth et al., 2001; Vodovotz et al., 1996; Wong et al., 2001). However, iNOS activity was not detected and its protein level was reduced or unchanged in AD brains in the present study. Because the supernatants were used for the radioenzymatic and colorimetric assays and Western blot, it is possible that iNOS-containing cells associated with the plaques were not present in the supernatants because of the high-speed centrifugation. To further address the iNOS issue in AD, immunohistochemistry was performed to compare the iNOS profile in SFG and CE between the NC-80 and AD-80 groups. In SFG, iNOS immunoreactivity was observed in neurons with moderate intensity and in astrocytes occasionally in the NC-80 group. In the AD group, however, there were intense iNOS-immunoreactive neurons and astrocytes, with some neurons displaying enhanced staining intensity and astrocytes forming clusters, which may indicate its presence in the tangle-bearing neurons and astrocytes surrounding SPs (Luth et al., 2001; Vodovotz et al., 1996). In CE, iNOS-immunoreactivity was mainly seen in the Purkinje cells in both groups, with no marked immunoreactive astrocytes. These immunohistochemical findings clearly demonstrate the increased presence of iNOS in the AD SFG, however, in agreement with the Western results, CE did not show an obvious increase in Alzheimer's cases.

In contrast to NOS, the present study found a marked increase in total arginase activity in the AD-80 group relative to the 2 control groups in all the 3 regions examined. Since there was no significant difference between the NC-60 and NC-80 groups, the increases in arginase activity appear to be AD-specific. Western blot data suggest that arginase II, rather than arginase I, is responsible for the activity changes in the superior frontal gyrus and hippocampus at least. A number of studies have investigated how arginase changes in AD brains, however with conflicting results. Colton et al. (2006), for example, reported increased arginase I mRNA expression (with no change in arginase II) in the AD frontal cortex, whereas Hansmannel et al. (2010) found increased

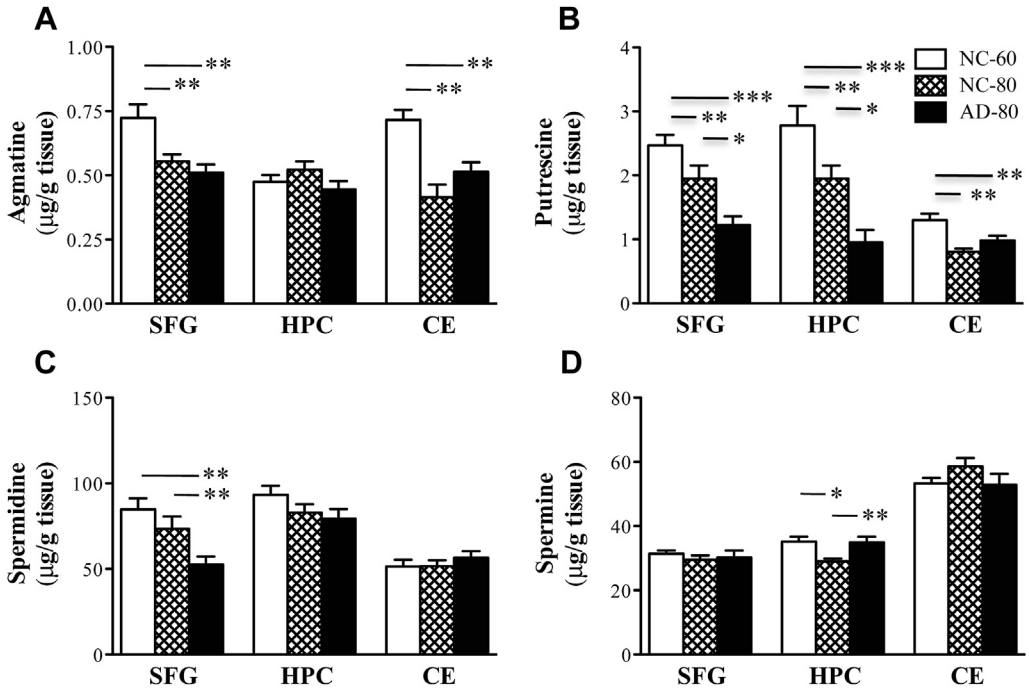

Fig. 6. Mean (±SEM) agmatine (A), putrescine (B), spermidine (C) and spermine (D) levels in the superior frontal gyrus (SFG), hippocampus (HPC), and cerebellum (CE) from neurologically normal cases with an average age of 60 (NC-60) or 80 (NC-80) years, or Alzheimer's disease cases with an average age of 80 years (AD-80). Asterisks indicate significant differences between groups at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. Abbreviation: SEM, standard error of the mean.

arginase II mRNA levels (with no change in arginase I) in the same region. L-arginine could also be metabolized by ADC to form agmatine (Fig. 1). Since ADC is labile and fresh tissue is required for measuring its activity (Li et al., 1994), we were unable to obtain the ADC activity data from the tissue that had been stored for several years (Tables 1–3).

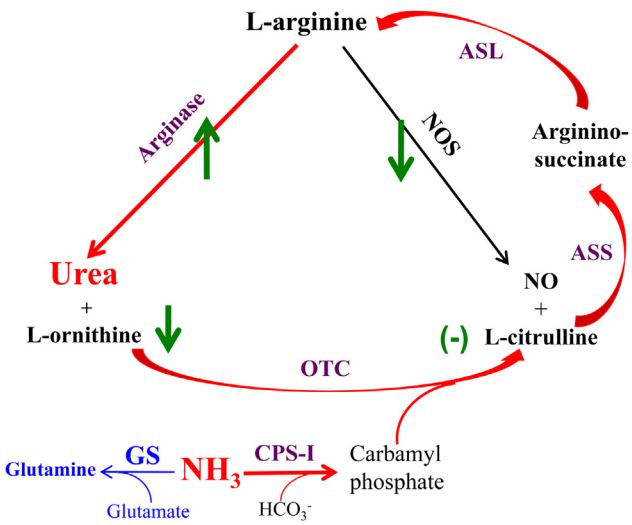

Fig. 7. The urea cycle in Alzheimer's brains. The urea cycle is comprised of carbamyl phosphate synthetase-I (CPS-I), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase to dispose of toxic ammonia (NH_3) in the nontoxic form of urea. In normal adult brains, the urea cycle is incomplete (hence inactive) because of the lack of OTC. Under physiological conditions, brain ammonia is metabolized to glutamine by glutamine synthetase (GS). The present study found markedly increased arginase activity and decreased NOS activity in AD brains that were however accompanied by dramatically reduced level of L-ornithine and insignificantly altered L-citrulline level (summarized by the green symbols). These findings suggest that L-ornithine is converted to L-citrulline by OTC in AD brains. Abbreviations: AD, Alzheimer's disease; NOS, nitric oxide synthetase.

It has been documented that NOS has an approximately 1000-fold greater affinity for L-arginine than arginase (Wu and Morris, 1998), and that endogenous agmatine levels in the mammalian brain are only about 1.5–3.0 nmol per gram of tissue (0.2–0.4 μg/g) (Li et al., 1994), suggesting the predominance of the NOS pathway under physiological situations. It is of interest to note the inverse relationship between total NOS and arginase activity in the AD-80 group in all 3 regions examined, which may reflect the competitive relationship between the 2 enzymes and a switch of L-arginine metabolism from the predominant NOS pathway to the arginase pathway during AD. It is currently unclear, however, whether the up-regulation of arginase is the cause or a consequence of altered NOS. Previous research has shown reduced availability of tetrahydrobiopterin (BH4, a key enzymatic co-factor for NO production) and increased level of asymmetric dimethylarginine (an endogenous NOS inhibitor) in AD and aging (Arlt et al., 2008; Foxton et al., 2007; Selley, 2003; Xiong et al., 2001), which may also contribute to the reduction of NOS activity to a certain extent. It is important to note that in both the NC-60 and NC-80 groups the cause of death in most cases was cardiovascular-related disorders, which are the known risk factors for AD (Kalaria et al., 2012; Orsucci et al., 2013). Given the role of NO, particularly that derived from eNOS, in cardiovascular and cerebrovascular function (Toda and Okamura, 2012; Tousoulis et al., 2012), certain degrees of cerebral eNOS dysfunction may be present in these cases.

4.2. AD- and age-related changes in L-arginine and its downstream metabolites

As illustrated in Fig. 1, L-arginine can be metabolized to form a number of bioactive molecules. The present study, therefore, compared the tissue concentrations of L-arginine and its 9 downstream metabolites in the superior frontal gyrus, hippocampus, and cerebellum across the 3 groups. For L-arginine, we found a significant increase in the AD SFG only. While the total NOS activity was

markedly reduced in the AD-80 and/or NC-80 groups, there were no significant differences between groups in L-citrulline (product of NOS) in all 3 regions examined. For L-ornithine (product of arginase), we found a dramatic reduction in the AD-80 group only for all the 3 areas, which obviously contradicts with AD-specific increase in arginase activity. In terms of agmatine, the levels were significantly decreased in SFG and CE in the NC-80 and AD-80 groups relative to the NC-60 group, suggesting an age-related reduction in agmatine.

It has been shown that L-ornithine can be channeled to form polyamines and glutamate and GABA (Wiesinger, 2001; Wu and Morris, 1998). The present study found markedly reduced putrescine levels with AD (in SFG and HPC) and age (in all 3 regions), mild changes in spermidine (reduced in the AD SFG), spermine (decreased in the NC-80 HPC), and glutamate (decreased and increased levels with AD in HPC and CE, respectively), and no changes in GABA. It has been well documented that physiological concentrations of polyamines are essential in maintaining normal cellular function (Alm and Oredsson, 2009; Igarashi and Kashiwagi, 2010; Wallace, 2009; Wallace et al., 2003). Polyamines interact with the NMDA receptors at their binding site and hence influence the receptor function (Igarashi and Kashiwagi, 2010; Rock and Macdonald, 1995). Additionally, there is evidence suggesting an important role of polyamines (particularly putrescine) in adult neurogenesis (Malaterre et al., 2004). It has been documented that agmatine stands at the crossroad of arginine metabolism to regulate the production of NO and polyamines (Halaris and Plietz, 2007; Piletz et al., 2013; Satriano, 2003) and is involved in learning and memory processing (Rushaidhi et al., 2013). Hence, altered agmatine and polyamine concentrations during AD and advanced aging may have important functional significance, which will be addressed in future studies.

It is of interest to emphasize the intriguing findings of increased arginase activity and decreased NOS activity in the AD brains that were however accompanied by reduced levels of the arginase metabolite L-ornithine and unaltered levels of the NOS metabolite L-citrulline. As there were marked decreases in putrescine and only mild changes in glutamate in AD brains, the data obviously do not support the up-regulation of arginase for the biosynthesis of polyamines and/or glutamate. It is noteworthy, however, that L-ornithine can also be metabolized by ornithine transcarbamylase (OTC) to form L-citrulline, which is then recycled to generate L-arginine by argininosuccinate synthetase and argininosuccinate lyase (Fig. 7; Wiesinger, 2001; Wu and Morris, 1998). Thus the pattern of results for L-ornithine and L-citrulline might be explained by an up-regulated OTC. Bensemain et al. (2009), interestingly, reported the expression of OTC in AD brains (but not in control brains) and a near 9-fold increase of OTC activity in the cerebrospinal fluid in AD cases relative to controls. OTC, argininosuccinate synthetase, argininosuccinate lyase, and arginase, along with carbamyl phosphate synthetase-I, are the 5 enzymes of the urea cycle (Fig. 7), which is an essential pathway in mammals to dispose of toxic ammonia in the nontoxic and readily excretable form of urea (Morris, 2002). Because the urea cycle is primarily expressed in the liver and normal brains lack OTC, brain ammonia is metabolized almost exclusively to glutamine by GS (Fig. 7; Felipo and Butterworth, 2002; Wiesinger, 2001). As ammonia is produced excessively in the brains of AD patients (Fisman et al., 1985; Seiler, 2002), it is likely that OTC is induced to cope with excessive ammonia. It is of interest to note that the present study found markedly increased glutamine levels in SFG and CE (a trend in HPC, $p = 0.053$) in the AD group only, which might be the consequence of excessive ammonia in AD brains. Future research is required to better understand the mechanism(s) and implication of the brain urea cycle in AD.

5. Conclusions

The present study, for the first time, demonstrates the effects of AD, as well as advanced aging, on the brain metabolic profile of L-arginine. There appear to be down-regulated NOS pathway (mainly nNOS and eNOS), with the superior frontal gyrus and hippocampus exhibiting AD- and age-related changes and the cerebellum only showing age-related alterations, and up-regulated arginase pathway (especially arginase II) specifically in AD brains. Immunohistochemistry confirmed the presence of iNOS in both AD and advanced aged brains, with intense immunoreactive neurons and astrocytes in the AD SFG. There were also AD- and age-related changes in the tissue concentrations of L-arginine and its 9 downstream metabolites in a metabolite- or region-specific manner. It has been documented that the cerebellum is resistant to amyloid angiopathy (Wegiel et al., 1999), and the AD cerebellum contains SPs but not NFTs (Larner, 1997). These findings suggest a unique pattern of AD pathology in the cerebellum, which may explain why the cerebellum has rather different AD-related neurochemical changes relative to the superior frontal gyrus and hippocampus.

It is important to note that our AD-80 cohort contained a highly heterogeneous population with large variations in both the disease status and the duration of symptoms (Table 3). Hence, some AD-specific changes observed in the present study need to be validated in future studies with larger sample sizes and less heterogenous populations. It is unclear at present whether altered arginine metabolism is a cause or a consequence of AD. There is evidence suggesting that NO may be an initiator of brain lesions during the development of AD—a neuronal energy crisis because of endothelial dysfunction during the advanced aging, together with other risk factors, triggers the progressive neurodegenerative process (Aliev et al., 2009; de la Torre and Stefano, 2000). Austin et al. (2010) demonstrated that endothelial NO played an important role in modulating amyloid precursor protein expression and processing (hence the production of Aβ) within the brain and cerebrovasculature. Furthermore, peptidylarginine deiminases (PAD2), an enzyme that metabolizes protein-arginine to protein-citrulline and ammonia, catalyses fibrillogenesis of Aβ (Mohlake and Whiteley, 2010), and citrullinated protein catalyzed by PAD2 is abnormally accumulated in AD brains (Ishigami et al., 2005). Future research is required to better understand the role of altered L-arginine metabolism in the pathogenesis and/or progression of AD.

Disclosure statement

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Health Research Council of New Zealand (10/170) and the Neurological Foundation of New Zealand. The authors express their appreciation to the Alzheimer's families in New Zealand for their invaluable assistance, and to the Neurological Foundation of New Zealand Human Brain Bank. They also thank Jocelyn Bullock and Marika Eszes in the Department of Anatomy with Radiology, University of Auckland, and the technical staff in the Department of Anatomy and School of Pharmacy, University of Otago, for their assistance.

References

Aliev, G., Palacios, H.H., Lipsitt, A.E., Fischbach, K., Lamb, B.T., Obrenovich, M.E., Morales, L., Gasimov, E., Bragin, V., 2009. Nitric oxide as an initiator of brain

lesions during the development of Alzheimer disease. Neurotox. Res. 16, 293–305.
Alm, K., Oredsson, S., 2009. Cells and polyamines do it cyclically. Essays Biochem. 46, 63–76.
Arlt, S., Schulze, F., Eichenlaub, M., Maas, R., Lehmbeck, J.T., Schwedhelm, E., Jahn, H., Böger, R.H., 2008. Asymmetrical dimethylarginine is increased in plasma and decreased in cerebrospinal fluid of patients with Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 26, 58–64.
Austin, S.A., Santhanam, A.V., Katusic, Z.S., 2010. Endothelial nitric oxide modulates expression and processing of amyloid precursor protein. Circ. Res. 107, 1498–1502.
Bensemain, F., Hot, D., Ferreira, S., Dumont, J., Bombois, S., Maurage, C.A., Huot, L., Hermant, X., Levillain, E., Hubans, C., Hansmannel, F., Chapuis, J., Hauw, J.J., Schraen, S., Lemoine, Y., Buee, L., Berr, C., Mann, D., Pasquier, F., Amouyel, P., Lambert, J.C., 2009. Evidence for induction of the ornithine transcarbamylase expression in Alzheimer's disease. Mol. Psychiatry 14, 106–116.
Braak, H., Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 82, 239–259.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A 87, 682–685.
Cho, D.H., Nakamura, T., Fang, J., Cieplak, P., Godzik, A., Gu, Z., Lipton, S.A., 2009. S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324, 102–105.
Colton, C.A., Mott, R.T., Sharpe, H., Xu, Q., Van Nostrand, W.E., Vitek, M.P., 2006. Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J. Neuroinflammation 3, 27.
de la Torre, J.C., 2012. Cerebral hemodynamics and vascular risk factors: setting the stage for Alzheimer's disease. J. Alzheimers Dis. 32, 553–567.
de la Torre, J.C., Stefano, G.B., 2000. Evidence that Alzheimer's disease is a microvascular disorder: the role of constitutive nitric oxide. Brain Res. Rev. 34, 119–136.
Ellison, D.W., Beal, M.F., Mazurek, M.F., Bird, E.D., Martin, J.B., 1986. A postmortem study of amino acid neurotransmitters in Alzheimer's disease. Ann. Neurol. 20, 616–621.
Feil, R., Kleppisch, T., 2008. NO/cGMP-dependent modulation of synaptic transmission. Handb. Exp. Pharmacol. 184, 529–560.
Felipo, V., Butterworth, R.F., 2002. Neurobiology of ammonia. Prog. Neurobiol. 67, 259–279.
Fisman, M., Gordon, B., Feleki, V., Helmes, E., Appell, J., Rabheru, K., 1985. Hyperammonemia in Alzheimer's disease. Am. J. Psychiatry 142, 71–73.
Forstermann, U., Sessa, W.C., 2012. Nitric oxide synthases: regulation and function. Eur. Heart J. 33, 829–837, 837a-837d.
Foxton, R.H., Land, J.M., Heales, S.J., 2007. Tetrahydrobiopterin availability in Parkinson's and Alzheimer's disease; potential pathogenic mechanisms. Neurochem. Res. 32, 751–756.
Gupta, N., Jing, Y., Collie, N.D., Zhang, H., Liu, P., 2012. Ageing alters behavioural function and brain arginine metabolism in male Sprague-Dawley rats. Neuroscience 226C, 178–196.
Halaris, A., Plietz, J., 2007. Agmatine : metabolic pathway and spectrum of activity in brain. CNS Drugs 21, 885–900.
Hansmannel, F., Sillaire, A., Kamboh, M.I., Lendon, C., Pasquier, F., Hannequin, D., Laumet, G., Mounier, A., Ayral, A.M., DeKosky, S.T., Hauw, J.J., Berr, C., Mann, D., Amouyel, P., Campion, D., Lambert, J.C., 2010. Is the urea cycle involved in Alzheimer's disease? J. Alzheimers Dis. 21, 1013–1021.
Hardy, J.A., Higgins, G.A., 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science 256, 184–185.
Igarashi, K., Kashiwagi, K., 2010. Modulation of cellular function by polyamines. Int. J. Biochem. Cell Biol. 42, 39–51.
Ishigami, A., Ohsawa, T., Hiratsuka, M., Taguchi, H., Kobayashi, S., Saito, Y., Murayama, S., Asaga, H., Toda, T., Kimura, N., Maruyama, N., 2005. Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. J. Neurosci. Res. 80, 120–128.
Jeynes, B., Provias, J., 2009. Significant negative correlations between capillary expressed eNOS and Alzheimer lesion burden. Neurosci. Lett. 463, 244–248.
Joshi, M.S., Ferguson Jr., T.B., Johnson, F.K., Johnson, R.A., Parthasarathy, S., Lancaster Jr., J.R., 2007. Receptor-mediated activation of nitric oxide synthesis by arginine in endothelial cells. Proc. Natl. Acad. Sci. U.S.A 104, 9982–9987.
Kalaria, R.N., Akinyemi, R., Ihara, M., 2012. Does vascular pathology contribute to Alzheimer changes? J. Neurol. Sci. 322, 141–147.
Knox, L.T., Jing, Y., Fleete, M.S., Collie, N.D., Zhang, H., Liu, P., 2011. Scopolamine impairs behavioural function and arginine metabolism in the rat dentate gyrus. Neuropharmacology 61, 1452–1462.
Larner, A.J., 1997. The cerebellum in Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 8, 203–209.
Law, A., Gauthier, S., Quirion, R., 2001. Say NO to Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res. Brain Res. Rev. 35, 73–96.
Leitch, B., Shevtsova, O., Reusch, K., Bergin, D.H., Liu, P., 2011. Spatial learning-induced increase in agmatine levels at hippocampal CA1 synapses. Synapse 65, 146–153.
Li, G., Regunathan, S., Barrow, C.J., Eshraghi, J., Cooper, R., Reis, D.J., 1994. Agmatine: an endogenous clonidine-displacing substance in the brain. Science 263, 966–969.
Liu, P., Chary, S., Devaraj, R., Jing, Y., Darlington, C.L., Smith, P.F., Tucker, I.G., Zhang, H., 2008a. Effects of aging on agmatine levels in memory-associated brain structures. Hippocampus 18, 853–856.
Liu, P., Collie, N.D., Chary, S., Jing, Y., Zhang, H., 2008b. Spatial learning results in elevated agmatine levels in the rat brain. Hippocampus 18, 1094–1098.
Liu, P., Gupta, N., Jing, Y., Zhang, H., 2008c. Age-related changes in polyamines in memory-associated brain structures in rats. Neuroscience 155, 789–796.
Liu, P., Jing, Y., Collie, N.D., Campbell, S.A., Zhang, H., 2011. Pre-aggregated Abeta(25-35) alters arginine metabolism in the rat hippocampus and prefrontal cortex. Neuroscience 193, 269–282.
Liu, P., Jing, Y., Collie, N.D., Chary, S., Zhang, H., 2009a. Memory-related changes in L-citrulline and agmatine in the rat brain. Hippocampus 19, 597–602.
Liu, P., Jing, Y., Zhang, H., 2009b. Age-related changes in arginine and its metabolites in memory-associated brain structures. Neuroscience 164, 611–628.
Liu, P., Smith, P.F., Appleton, I., Darlington, C.L., Bilkey, D.K., 2003a. Nitric oxide synthase and arginase in the rat hippocampus and the entorhinal, perirhinal, postrhinal, and temporal cortices: regional variations and age-related changes. Hippocampus 13, 859–867.
Liu, P., Smith, P.F., Appleton, I., Darlington, C.L., Bilkey, D.K., 2003b. Regional variations and age-related changes in nitric oxide synthase and arginase in the subregions of the hippocampus. Neuroscience 119, 679–687.
Liu, P., Smith, P.F., Appleton, I., Darlington, C.L., Bilkey, D.K., 2005. Hippocampal nitric oxide synthase and arginase and age-associated behavioral deficits. Hippocampus 15, 642–655.
Liu, P., Zhang, H., Devaraj, R., Ganesalingam, G.S., Smith, P.F., 2010. A multivariate analysis of the effects of aging on glutamate, GABA and arginine metabolites in the rat vestibular nucleus. Hear. Res. 269, 122–133.
Lundberg, J.O., Weitzberg, E., Gladwin, M.T., 2008. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat. Rev. Drug Discov. 7, 156–167.
Luth, H.J., Holzer, M., Gartner, U., Staufenbiel, M., Arendt, T., 2001. Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. Brain Res. 913, 57–67.
Malaterre, J., Strambi, C., Aouane, A., Strambi, A., Rougon, G., Cayre, M., 2004. A novel role for polyamines in adult neurogenesis in rodent brain. Eur. J. Neurosci. 20, 317–330.
Malinski, T., 2007. Nitric oxide and nitroxidative stress in Alzheimer's disease. J. Alzheimers Dis. 11, 207–218.
Marques, F., Sousa, J.C., Sousa, N., Palha, J.A., 2013. Blood-brain-barriers in aging and in Alzheimer's disease. Mol. Neurodegener. 8, 38.
Milsom, A.B., Fernandez, B.O., Garcia-Saura, M.F., Rodriguez, J., Feelisch, M., 2012. Contributions of nitric oxide synthases, dietary nitrite/nitrate, and other sources to the formation of NO signaling products. Antioxid. Redox Signal 17, 422–432.
Mohlake, P., Whiteley, C.G., 2010. Arginine metabolising enzymes as therapeutic tools for Alzheimer's disease: peptidyl arginine deiminase catalyses fibrillogenesis of beta-amyloid peptides. Mol. Neurobiol. 41, 149–158.
Morris Jr., S.M., 2002. Regulation of enzymes of the urea cycle and arginine metabolism. Annu. Rev. Nutr. 22, 87–105.
Morrison, L.D., Cao, X.C., Kish, S.J., 1998. Ornithine decarboxylase in human brain: influence of aging, regional distribution, and Alzheimer's disease. J. Neurochem. 71, 288–294.
Morrison, L.D., Kish, S.J., 1995. Brain polyamine levels are altered in Alzheimer's disease. Neurosci. Lett. 197, 5–8.
Norris, P.J., Faull, R.L., Emson, P.C., 1996. Neuronal nitric oxide synthase (nNOS) mRNA expression and NADPH-diaphorase staining in the frontal cortex, visual cortex and hippocampus of control and Alzheimer's disease brains. Brain Res. Mol. Brain Res. 41, 36–49.
Orsucci, D., Mancuso, M., Ienco, E.C., Simoncini, C., Siciliano, G., Bonuccelli, U., 2013. Vascular factors and mitochondrial dysfunction: a central role in the pathogenesis of Alzheimer's disease. Curr. Neurovasc. Res. 10, 76–80.
Piletz, J.E., Aricioglu, F., Cheng, J.-T., Fairbanks, C.A., Gilad, V.H., Haenisch, B., Halaris, A., Hong, S., Lee, J.E., Li, J., Liu, P., Molderings, G.J., Rodrigues, A.L.S., Satriano, J., Seong, G.J., Wilcox, G., Wu, N., Gilad, G.M., 2013. Agmatine: clinical applications after 100 years in translation. Drug Discov Today 18, 880–893.
Provias, J., Jeynes, B., 2008. Neurofibrillary tangles and senile plaques in Alzheimer's brains are associated with reduced capillary expression of vascular endothelial growth factor and endothelial nitric oxide synthase. Curr. Neurovasc. Res. 5, 199–205.
Reis, D.J., Regunathan, S., 2000. Is agmatine a novel neurotransmitter in brain? Trends Pharmacol. Sci. 21, 187–193.
Rock, D.M., Macdonald, R.L., 1995. Polyamine regulation of N-methyl-D-aspartate receptor channels. Annu. Rev. Pharmacol. Toxicol. 35, 463–482.
Rushaidhi, M., Jing, Y., Zhang, H., Liu, P., 2013. Participation of hippocampal agmatine in spatial learning: an in vivo microdialysis study. Neuropharmacology 65, 200–205.
Santhanam, A.V., Viswanathan, S., Dikshit, M., 2007. Activation of protein kinase B/Akt and endothelial nitric oxide synthase mediates agmatine-induced endothelium-dependent relaxation. Eur. J. Pharmacol. 572, 189–196.
Satriano, J., 2003. Agmatine: at the crossroads of the arginine pathways. Ann. N.Y. Acad. Sci. 1009, 34–43.
Seiler, N., 2002. Ammonia and Alzheimer's disease. Neurochem. Int. 41, 189–207.
Selkoe, D.J., 2011. Alzheimer's disease. Cold Spring Harb. Perspect. Biol. 3; pii: a004457.

Selley, M.L., 2003. Increased concentrations of homocysteine and asymmetric dimethylarginine and decreased concentrations of nitric oxide in the plasma of patients with Alzheimer's disease. Neurobiol. Aging 24, 903–907.
Seo, S., Liu, P., Leitch, B., 2011. Spatial learning-induced accumulation of agmatine and glutamate at hippocampal CA1 synaptic terminals. Neuroscience 192, 28–36.
Serrano-Pozo, A., Frosch, M.P., Masliah, E., Hyman, B.T., 2011. Neuropathological alterations in Alzheimer disease. Cold Spring Harb. Perspect. Med. 1, a006189.
Sun, J., Zhang, X., Broderick, M., Fein, H., 2003. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors 3, 276–284.
Susswein, A.J., Katzoff, A., Miller, N., Hurwitz, I., 2004. Nitric oxide and memory. Neuroscientist 10, 153–162.
Tapiero, H., Mathe, G., Couvreur, P., Tew, K.D., 2002. II. Glutamine and glutamate. Biomed. Pharmacother. 56, 446–457.
Thorns, V., Hansen, L., Masliah, E., 1998. nNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. Exp. Neurol. 150, 14–20.
Toda, N., Okamura, T., 2012. Cerebral blood flow regulation by nitric oxide in Alzheimer's disease. J. Alzheimers Dis. 32, 569–578.
Tousoulis, D., Kampoli, A.M., Tentolouris, C., Papageorgiou, N., Stefanadis, C., 2012. The role of nitric oxide on endothelial function. Curr. Vasc. Pharmacol. 10, 4–18.
Trushina, E., Dutta, T., Persson, X.M., Mielke, M.M., Petersen, R.C., 2013. Identification of altered metabolic pathways in plasma and CSF in mild cognitive impairment and Alzheimer's disease using metabolomics. PLoS One 8, e63644.
Trushina, E., Mielke, M.M., 2013. Recent advances in the application of metabolomics to Alzheimer's disease. Biochim. Biophys. Acta; pii: S0925-4439(13)00223-8.
Tumani, H., Shen, G., Peter, J.B., Bruck, W., 1999. Glutamine synthetase in cerebrospinal fluid, serum, and brain: a diagnostic marker for Alzheimer disease? Arch. Neurol. 56, 1241–1246.
Vodovotz, Y., Lucia, M.S., Flanders, K.C., Chesler, L., Xie, Q.W., Smith, T.W., Weidner, J., Mumford, R., Webber, R., Nathan, C., Roberts, A.B., Lippa, C.F., Sporn, M.B., 1996. Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. J. Exp. Med. 184, 1425–1433.
Waldvogel, H.J., Bullock, J.Y., Synek, B.J., Curtis, M.A., van Roon-Mom, W.M., Faull, R.L., 2008. The collection and processing of human brain tissue for research. Cell Tissue Bank 9, 169–179.
Waldvogel, H.J., Curtis, M.A., Baer, K., Rees, M.I., Faull, R.L., 2006. Immunohistochemical staining of post-mortem adult human brain sections. Nat. Protoc. 1, 2719–2732.
Wallace, H.M., 2009. The polyamines: past, present and future. Essays Biochem. 46, 1–9.
Wallace, H.M., Fraser, A.V., Hughes, A., 2003. A perspective of polyamine metabolism. Biochem. J. 376, 1–14.
Wegiel, J., Wisniewski, H.M., Dziewiatkowski, J., Badmajew, E., Tarnawski, M., Reisberg, B., Mlodzik, B., De Leon, M.J., Miller, D.C., 1999. Cerebellar atrophy in Alzheimer's disease-clinicopathological correlations. Brain Res. 818, 41–50.
Wiesinger, H., 2001. Arginine metabolism and the synthesis of nitric oxide in the nervous system. Prog. Neurobiol. 64, 365–391.
Xiong, Y., Yuan, L.W., Deng, H.W., Li, Y.J., Chen, B.M., 2001. Elevated serum endogenous inhibitor of nitric oxide synthase and endothelial dysfunction in aged rats. Clin. Exp. Pharmacol. Physiol. 28, 842–847.
Wong, A., Luth, H.J., Deuther-Conrad, W., Dukic-Stefanovic, S., Gasic-Milenkovic, J., Arendt, T., Munch, G., 2001. Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease. Brain Res. 920, 32–40.
Wu, G., Morris, S.M., 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336, 1–17.
Yew, D.T., Wong, H.W., Li, W.P., Lai, H.W., Yu, W.H., 1999. Nitric oxide synthase neurons in different areas of normal aged and Alzheimer's brains. Neuroscience 89, 675–686.
Yi, J., Horky, L.L., Friedlich, A.L., Shi, Y., Rogers, J.T., Huang, X., 2009. L-arginine and Alzheimer's disease. Int. J. Clin. Exp. Pathol. 2, 211–238.
Zhou, L., Zhu, D.Y., 2009. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric Oxide 20, 223–230.
Zolman, J.F., 1993. Biostatistics: Experimental Design and Statistical Inference. Oxford University Press, Oxford, UK.